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Effects of temperature and tissue type on *Chrysomya rufifacies* (Diptera: Calliphoridae) (Macquart) development

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ABSTRACT

The hairy maggot blow fly, *Chrysomya rufifacies* (Diptera: Calliphoridae), is a forensically important fly often encountered on human and other vertebrate remains in temperate and tropic regions throughout the world including Australia, Asia, Central America and North America. *C. rufifacies* was reared under controlled laboratory conditions on three muscle types (i.e., porcine, equine and canine) at three temperatures (i.e., 20.8, 24.8 and 28.3 °C). Rate of larval weight gain across time was statistically significant between muscle types ($P \leq 0.0001$) and approaching significance across time between temperatures ($P = 0.0511$). This research represents the first development study for *C. rufifacies* from central Texas, USA and the first study to examine the impact of tissue type on its development. Furthermore, these data, when compared to those available in the literature, indicate developmental differences that could be due to genetic differences in populations or possibly methods employed during the studies. Caution should be emphasized when applying development data for this species from one region to forensic investigations in other ecoregions as such differences in development based on tissue fed upon by larvae, population genetics, and methodologies used in the studies could represent error in estimating the time of colonization.

1. Introduction

Blow fly (Diptera: Calliphoridae) development is a quantitative trait; a trait that is known to vary due to genetic and environmental factors [1]. Studies on blow flies have demonstrated this variation [2–4], but more work is needed within blow flies to understand how single populations and multiple genotypes of the same species respond to such conditions [5].

Temperature is a well-recognized abiotic factor that affects blow fly development. In most cases, warmer temperatures accelerate development while cooler temperatures have an inverse impact. This relationship has been documented in past growth studies on blow flies at varying temperatures [6–9]. However, the amount of daily variation in temperature (i.e., temperature fluctuation) experienced by blow fly immatures can also influence their rate of development, with cyclic temperatures increasing or decreasing development times, depending on the species [7–10].

Type of tissue fed to immature blow flies also impacts their size and development rate [4,11]. Clark et al. [11] determined that *Lucilia sericata* (Meigen) (Diptera: Calliphoridae) larvae reared on porcine tissue grew faster and larger than those on bovine tissue, while larvae fed lung and heart of both tissue types grew faster and larger than larvae fed liver. Kaneshrajah and Turner [4] recorded similar results for *Calliphora vicina* Robineau-Desvoidy (Diptera: Calliphoridae) larvae with those fed pig lung, kidney, heart or brain growing faster and larger than those provided pig liver. Tarone and Foran [2] showed that, even when fed only beef liver, *L. sericata* larvae possess the potential to develop at different rates depending on the experimental conditions (specifically factors affecting liver moisture and the condition of the pupation substrate). A Texas population of *Cochliomyia macellaria* (Fabricius) (Diptera: Calliphoridae), native to the Americas, was studied as to the effects of temperature and tissue type as it relates to its development [12]. Temperature rather than tissue type, was determined to significantly impact *Co. macellaria* development ($P < 0.05$).

Chrysomya rufifacies (Macquart) (Diptera: Calliphoridae) is an invasive blow fly species from Australia, New Zealand, New Caledonia, Samoa, Marquesas Island, Fiji, Tonga, Java, India, Ceylon (currently Sri Lanka) [13], Thailand [14], Pakistan and Iran [15]. It

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has been recorded in Japan, Baluchistan, Sumatra, Celebes, Tasmania, New Hebrides, Saipan, Solomon Islands, Society Islands [16], Malaysia, Calcutta [17] and was first introduced to Central America (Costa Rica) in 1978 where is has since moved to Puerto Rico, Guatemala, Mexico [18], and Argentina [19]. Making its way up to North America it has steadily expanded its range beyond its once-believed environmental tolerance [18,20]. *C. rufifacies* has been documented throughout the contiguous United States, from California to Florida, Hawaii [18] and as far north as southeastern Ontario, Canada [18,20]. *C. rufifacies*, like *Co. macellaria*, is commonly collected from animal remains in central Texas [21] and is also frequently encountered on deceased humans as well (Jeffery K. Tomberlin, unpublished data).

Both *C. rufifacies* and *Co. macellaria* have been hypothesized to be ecological counterparts [22]; however each fly has evolved in different ecological conditions. As *C. rufifacies* is native to habitats that experience minimal temperature variability (i.e., tropical region), this may have led to flies being less plastic (range of development time responses) to variations in temperature while *Co. macellaria* may exhibit greater developmental plasticity as it experiences a much more variable temperature range (i.e., temperate region). Additionally, the tropics are renowned for high species diversity [23], indicating that *C. rufifacies* may have evolved under conditions that require the ability to survive on numerous types of carrion. If these fly species truly are ecological counterparts not only will they share the same resources and environments, which have been documented previously in the literature [24], but they will also respond similarly to abiotic factors.

The objectives of this study were to determine the impact of temperature and muscle type on the development of a single population of *C. rufifacies*. We hypothesize that time for each stage of development will be affected by the larval rearing substrate (muscle type) as well as the temperatures to which the flies were exposed to throughout their life cycles.

2. Materials and methods

2.1. Fly source

C. rufifacies larvae (>500 individuals) were collected from decomposing animal remains located in College Station, TX, USA during July and October of 2008, May of 2009 and August of 2011 and subsequent F_1 generations used for necessary experiments at the collection times. Larvae were brought to the Texas A&M University Forensic Laboratory for Investigative Entomological Sciences (FLIES Facility) to initiate colonies. Resulting adult flies were held in multiple 30 × 30 × 30 cm BioQuip® (Rancho Dominguez, CA, USA) lumite screen collapsible cages in the FLIES Facility (~24.4 °C, 50% RH and 14:10L:D). Adult flies were provided a 50:50 mixture of table sugar and powdered milk or honey, as well as cotton balls soaked with deionized water (dH_2O) ad libitum.

2.2. Tissue source

Striated muscle tissue from three vertebrate animal species was used as a development medium in this study. Canine (*Canis lupus familiaris* Linnaeus) muscle tissue was obtained through an Austin-area (Austin, TX, USA) veterinarian from three separate canines (replicates). Equine (*Equus ferus* Boddaert) muscle tissue was donated by the Texas A&M University Veterinary Diagnostic Laboratory, College Station, TX, USA from three separate equines (replicates). Porcine (*Sus scrofa* L.) muscle tissue was obtained from a local grocery store from multiple packages of lean pork chops and separated into three groups (replicates). Bovine liver was obtained from an on campus meat processing plant (E.M. "Manny" Rosenthal Meat Science and Technology Center, College Station, TX, USA) and used as an oviposition medium and for rearing immature flies. For each animal and pork chop package, muscle tissue samples were placed in individual Ziploc bags, labeled and stored in a −20 °C freezer until use in the experiment. Muscle tissue acquisition protocols were approved by the Animal Welfare Assurance Program at Texas A&M University.

2.3. Development

Methods for the development study were adapted from Byrd and Butler [8] and identical to those used in the Boatright and Tomberlin [12] study. Adults (7–10 d) from the F_1 generation were provided with approximately 200 g fresh beef (bovine) liver as an oviposition site. Hourly observations were made for egg clutches. Egg clutches less than one hour old were placed in dH_2O, and disaggregated with a camel hair brush to randomize all female egg clutches and account for cohort variation. For each temperature treatment individual plastic BioQuip® mosquito-breeding container bottoms (10 [*h*] × 12 cm [*w*]) were placed into an individual sterilite plastic shoe box container (35 [*l*] × 20 [*w*] × 13 cm [*h*]) (Townsend, MA, USA). Each plastic shoe box contained approximately 500 ml (850 g) of sand (Quikrete Premium Play Sand, Atlanta, GA, USA) as pupation medium. The mosquito-breeding container was placed on top of the sand in the center of the shoebox. Each mosquito-breeding container held 200 g of porcine, canine, or equine muscle tissue which had been cubed (~3 cm^3 or ~25 g). Approximately 200 eggs, representing multiple clutches, were placed on a moistened filter paper to prevent desiccation and then placed on the respective cubed muscle tissue in an order determined using a random number generator. Egg number was determined gravimetrically with an Adventure-Pro AV64 Ohaus scale (Pine Brook, NJ, USA). The three replicates of each muscle tissue were placed in three stand-up environmental chambers (136LLVL Percival Scientific Inc., Perry, IA, USA) set at 21, 24, or 27 °C with 14:10L:D and 75–80% RH using a Latin square design which assigns each replicate to one of the three shelves without overlaps in animal tissue positions. A hobo data logger Onset (Onset Co., Pocasset, MA, USA) hobo U12-006 placed inside of each environmental chamber with probes placed on each of the three levels of the chamber to record temperature hourly.

Eggs were monitored hourly for hatch; thereafter, observations were made every 12 h. During each observation after egg hatch, three larvae of the visible cohort that approximated the largest larvae were collected, placed in hot water at approximately 100 °C for 30 s [25] and then measured as described below. Life stage, larval weight and length were recorded for each larva sampled as well as stage duration. Larvae were weighed using the scale previously described and length measured in millimeters with a Meiji Techno EMZ-8TR microscope (Santa Clara, CA, USA) and ruler. Larvae from each replicate were sampled until the cohorts had reached the pupal stage. For each replicate of muscle type, pupae were collected when they represented the oldest development stage present. The initial 30 pupae observed were sampled. Each of these pupae was placed individually in a 35 ml plastic container (Jetware, Hatfield, PA, USA) with approximately 10 ml of sand. Containers with pupae were labeled, returned to the appropriate growth chamber, and monitored for adult emergence. Time, date, and sex were recorded for each emergent adult. Resulting adults were provided 0.20 ml distilled water, every 24 h, via a 1 ml Kendall Monoject SoftPack Insulin Syringe (Mansfield, MA, USA) inserted through the lid and adult longevity was recorded. Stage duration was determined by observing when the first time an

instar was observed to the next observation when no individuals of that instar were collected in the sample.

A preliminary study was conducted in August 2011 to determine time to complete the egg stage at the three temperatures studied. Two hundred eggs collected from F_1 generation adults (same methods as above) were separated and weighed gravimetrically, placed on moistened filter paper to prevent desiccation and replicated six times. Care was taken to monitor how long eggs spent at room temperatures during weighing and at what point they were placed in their respective temperatures. Eggs were monitored hourly until hatch.

2.4. Statistics

A split plot design analysis of covariance (ANCOVA) was used to analyze the development data (SAS 9.2 for Windows, Carry, NC, USA) to determine the influence temperature and muscle types have on weight and length over time for each technical replicate. The whole plot represented muscle type and the split plot represented temperature with muscle tissue set up as a random factor. The split plot design accounted for the variation which occurs in the data on account of having three temperatures nested within each of the muscle types being tested. Treatment effects were considered significant when the corresponding *P*-values were <0.05. As environmental chambers were not replicated for this study to confirm that differences, if any, observed would be from only temperature and not minor differences between environment chambers the authors infer temperature is the sole responsibility [26].

3. Results

Larvae failed to reach the third instar in one of the canine muscle replicates. Therefore, results presented for canine are based on an $N = 2$. Mean weight over time as well as length over time are plotted for porcine, equine and canine muscle types (Figs. 1A–C and 2A–C respectively). Muscle type was not a statistically significant predictor of weight ($F_2 = 0.41$; $P = 0.6835$) or length ($F_2 = 0.03$; $P = 0.9725$). Weight or length across temperatures did not differ significantly (weight; $F_2 = 0.01$; $P = 0.9899$: length; $F_2 = 0.33$; $P = 0.7234$). Time was a statistically significant predictor ($P \leq 0.0001$) for both weight and length. A significant interaction between time and muscle type was observed for weight ($F_2 = 15.87$; $P \leq 0.0001$) and approached significance for length ($F_2 = 2.49$; $P = 0.0847$). The interaction between muscle type and temperature was not observed to be a significant predictor for weight ($F_4 = 0.03$; $P = 0.9981$) or length ($F_4 = 0.04$; $P = 0.9959$). An interaction between time and temperature approached significance with weight ($F_2 = 3.01$; $P = 0.0511$) but not length ($F_2 = 1.61$; $P = 0.2013$). The three way interaction between muscle type, temperature and time was not significant when predicting weight ($F_4 = 0.16$; $P = 0.9594$) or length ($F_4 = 0.25$; $P = 0.9122$).

Hours spent in each developmental stage were determined. Hours for egg hatch at the three temperatures are presented in

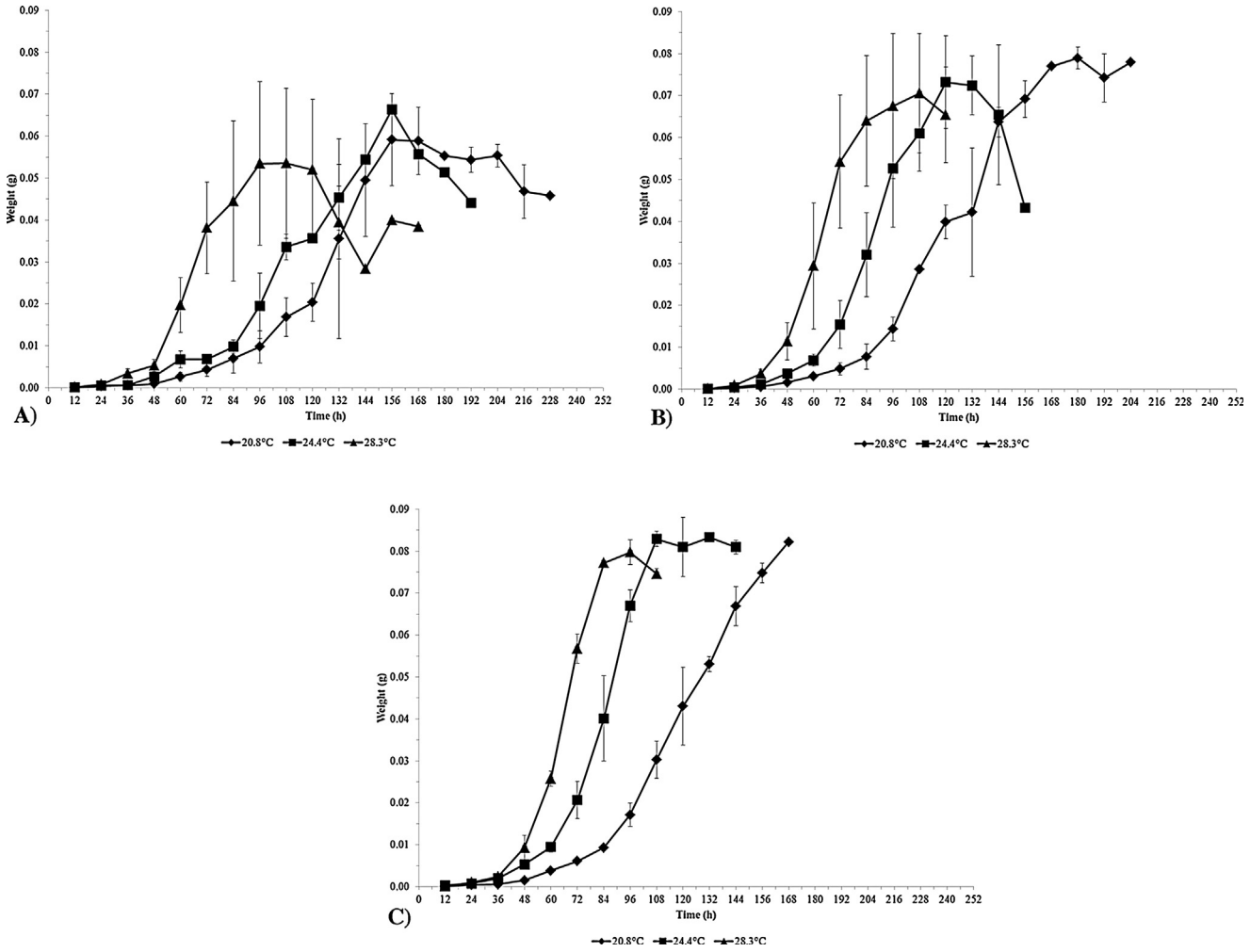

Fig. 1. *C. rufifacies* larval weight (g) ± SE developing at three temperatures on (a) porcine ($N = 3$), (b) equine ($N = 3$) and (c) canine ($N = 2$) muscle.

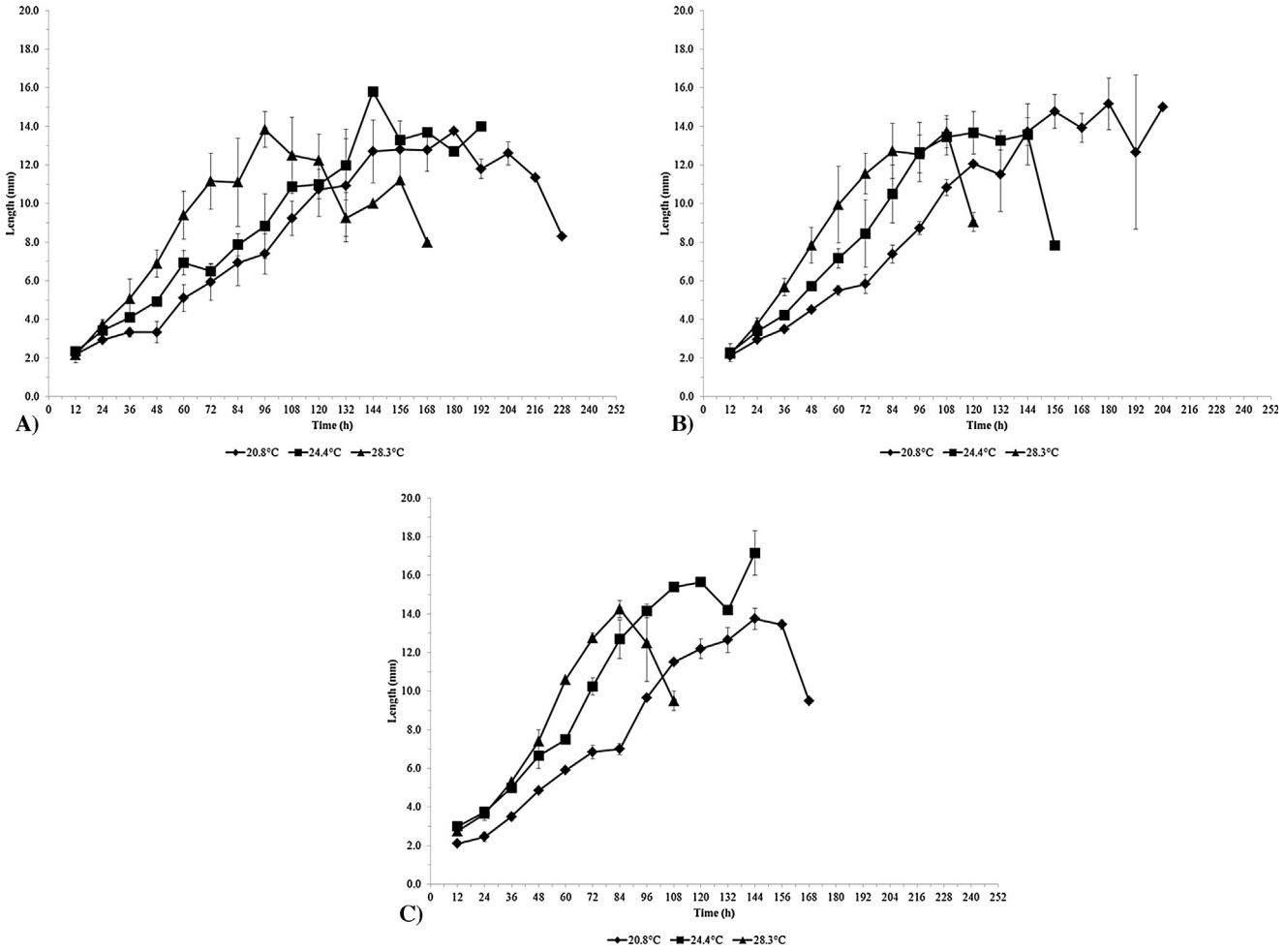

Fig. 2. *C. rufifacies* larval length (mm) ± SE developing at three temperatures on (a) porcine ($N = 3$), (b) equine ($N = 3$) and (c) canine ($N = 2$) muscle.

Table 1
Mean hours ± SE ($N^a = 3$) needed for *C. rufifacies* to finish the egg stage at three temperatures.

Temperature (°C)	Egg (h)
28.3	15.7 ± 0.7
24.4	20.5 ± 0.2
20.8	25.2 ± 0.2

[a] N = replicate containers.

Table 1. Results for stage durations are given in Table 2. Larvae needed 28 h longer to complete development to the adult stage (Table 2, First instar + Second instar + Third instar + Pupal times combined) on porcine and equine muscle tissue compared to the canine muscle tissue at the warmest temperature (28.3 °C). At 24.4 °C larvae on the porcine muscle tissue took the greatest amount of time to complete development to the adult stage (Table 2, First instar + Second instar + Third instar + Pupal times combined) by 12 h compared to the canine muscle and 20 h compared to the equine muscle. At the coolest temperature (20.8 °C) larvae on the porcine muscle tissue again took the longest amount of time to complete development to the adult stage (Table 2, First instar + Second instar + Third instar + Pupal times combined) by 44 h versus the equine muscle and 78 h versus the canine muscle. The largest larvae (length and weight) were recorded for the canine muscle (17.15 mm and 0.0833 g) at 24.4 °C,

Table 2
Mean hours ± SE ($N^a = 3$) needed for *C. rufifacies* to complete each stage of development when raised on three muscle types and at three temperatures.

Muscle type	Temperature (°C)	First instar	Second instar	Third instar	Pupa	Adult
Porcine	28.3	36.0 ± 6.9A[b]	32.0 ± 4.0A	92.0 ± 8.0A	84.0 ± 0.0A	32.0 ± 8.0A
	24.4	36.0 ± 0.0A	44.0 ± 14.4A	92.0 ± 26.2A	128.0 ± 4.0B	36.0 ± 6.9A
	20.8	52.0 ± 4.0A	64.0 ± 24.3A	120.0 ± 12.0A	172.0 ± 4.0 C	72.0 ± 6.9B
Equine	28.3	32.0 ± 4.0A	24.0 ± 0.0A	84.0 ± 0.0A	104.0 ± 8.0A	24.0 ± 0.0A
	24.4	40.0 ± 4.0A	32.0 ± 4.0A	88.0 ± 4.0A	120.0 ± 12.0A	52.0 ± 4.0AB
	20.8	48.0 ± 0.0A	40.0 ± 4.0A	112.0 ± 14.4A	164.0 ± 4.0B	68.0 ± 10.6B
Canine	28.3	36.0 ± 0.0A	18.0 ± 6.0A	72.0 ± 0.0A	90.0 ± 6.0A	24.0 ± 0.0A
	24.4	36.0 ± 0.0A	30.0 ± 6.0A	96.0 ± 0.0A	126.0 ± 6.0AB	60.0 ± 0.0AB
	20.8	48.0 ± 0.0A	42.0 ± 6.0A	90.0 ± 6.0A	150.0 ± 6.0B	84.0 ± 0.0B

No significant ($P < 0.05$) difference in development for larvae placed on different muscle types but at the same temperature was observed.
[a] N = replicate containers.
[b] Uppercase letters indicate significant ($P < 0.05$) difference in development for larvae placed on the same muscle types between temperatures.

porcine muscle (15.8 mm and 0.0663 g) at 24.4 °C and equine muscle (15.17 mm and 0.0789 g) at 20.8 °C.

4. Discussion

Methods for collecting development data and the way data are recorded is variable (e.g., stage specific, time to pupal stage or time to adult stage). Development data for *C. rufifacies* has been recoded previously for a Hawaii, USA population [27], Thailand population [28], and two unknown populations, possibly North American [9,29]. Excluding the current study, a single data set giving stage durations for *C. rufifacies* without larval replacement has been conducted in the United States [8]. We attempted to conduct our research using similar temperatures (20.8, 24.4 and 28.3 °C) as the Byrd and Butler [8] study (21.1, 25 and 26.7 °C) but the lower (21.1 °C) and higher (26.7 °C) temperatures in their study were run with a cyclic amplitude of 5.5 °C. All developmental stages at each temperature in the Byrd and Butler [8] study required less time to complete development than observed in this study except for the third instar stage when reared at the constant 25 °C temperature. In their case, the larvae needed an additional 14 h to complete the third instar stage than the average we observed, but this difference is encompassed in the standard error range and does not take into account the observation scale of 12 h employed in both of these studies.

In contrast to what Greenberg [9] proposed, the constant and cyclic temperature times to pupation being similar, we determined in all cases the larvae developed slower than what was recorded by Byrd and Butler [8] under these conditions. Larvae reared on porcine tissue in our experiments, which is the same tissue used in the Byrd and Butler [8] study, needed 40–70 h more to complete development. This relationship has been demonstrated previously for another blow fly species. A comparison between a central Texas population [12] and a Florida population [7] of *Co. macellaria* at 25 °C determined the Texas population also required more time (~95 h) to complete development than the Florida population, both of which were run at a constant temperature and on porcine tissue.

The discrepancies in development times between the population examined in this study and the Florida population may be due to genetic variation in *C. rufifacies*. However, in our study only a single population was examined and the differences seen in time to developmental stage completion across temperature could be an example of environmental plasticity. Genetic variation in development time has been examined previously for *L. sericata* from several populations [3,30]. Gallagher et al. [3] observed two populations from California, USA and one population from Massachusetts, USA in three environments (16.0, 26.0 and 36.0 °C temperatures). They observed more rapid development (~26 h) for flies from the Massachusetts population than in the California populations only in the intermediate temperature (26.0 °C) examined. Tarone and Foran [2] also examined one population from California, USA, one population from Michigan, USA and one population from West Virginia, USA in two environments (20.0 and 35.5 °C temperatures). West Virginia flies exhibited significantly smaller larvae (length and weight) whereas California, USA larvae were larger, though all distributions exhibited considerable overlap with one another [31].

Differences in development could also be due to variation in experimental design like those present between our study and the Florida study. Tarone and Foran [2] have demonstrated that altering experimental set up (e.g., larval feeding duration, food-substrate barriers and pupation substrate medium/method) can influence development times within populations of *L. sericata*. By varying environmental rearing conditions larval development times ranged from 329.0 to 505.5 h, which encompassed observed larval development times in the published literature for *L. sericata*. Several design differences were present between our study and the Florida study [8]. For example, the blow fly egg to tissue weight ratio varied between the studies. We placed 200 eggs on 200 g of porcine tissue while they placed 100 eggs on 200 g of porcine tissue. Their study used a ratio of 1 maggot/2 g of tissue to eliminate a possible effect of heat generation by the larvae. Had this been the case in our study we would expect faster development as noticed in other studies on larval maggot mass heat generation [27,32].

In our study we used a 14:10L:D cycle while Byrd and Butler [8] had a 12:12L:D for the cyclic temperature regimes and a 24:0L:D. Nabity et al. [33] have demonstrated that constant light (24:0L:D) in the black blow fly, *Phormia regina* Meigen (Diptera: Calliphoridae), led to an approximately 48 h longer development time when compared to a cyclic light cycle (12:12L:D) across various temperatures studied.

The Florida study also had cyclic temperature regimes (as previously discussed) while this study was conducted with the growth chambers set at a constant temperature. Fluctuating temperatures have been shown to have various effect on larval growth with some species taking a longer time to develop (*C. rufifacies*, *Co. macellaria*, *P. regina*, *Phaenicia* (*Lucilia*) *sericata*, *Calliphora vomitoria* L. (Diptera: Calliphoridae) and *Ca. vicina*) and others taking less time to develop (*Sarcophaga argyrostoma* Robineau-Desvoidy (Diptera: Sarcophagidae) and *Lucilia illustris* Meigen (Diptera: Calliphoridae)) [9,10]. For *C. rufifacies*, Greenberg [9] found that at a constant temperature of 22.5 °C were faster (358–366 h) than flies reared at a fluctuating temperature (16–29 °C, average 22.5 °C), 387–402 h, by 9% but this was found to not be significant.

Protocols for collecting larval samples also varied between studies. Byrd and Butler [8] collected two of the largest larvae every 12 h, boiled them and placed them in 75% ethanol to be measured later. In our study we collected three of the largest larvae every 12 h, boiled the larvae and then measured them soon after that. Differences have been observed in the preservation of larvae after being boiled and placed in ethanol [25]. One or a combination of these factors could attribute to the differences observed between these two studies.

C. rufifacies growth was more impacted by the tissue provided rather than the temperature experienced. This trend was opposite of what was observed for a *Co. macellaria* population [12] even though both species of flies were collected in the same area and around the same time. These differences in response to uncontrollable factors in the field could lead to the coexistence of the two species in this eco-region, with one species at the advantage when temperatures fluctuate and the other at the advantage when resource types are more varied.

This research is also important to the field of forensic entomology, which is the application of arthropod science in the judicial system. Forensic entomologists assist in criminal cases by estimating the time of insect colonization of human, or other animals remains [34]. In order to make these estimates, forensic entomologists rely on laboratory development data for the species in question. Given that colonization by many of these arthropods occurs after death, these estimates are synonymous with the minimum postmortem interval (mPMI) [35]. The need for development data for these forensically important species from various eco-regions is necessary as they might be significantly different [2,12]. Accounting for this variation could help reduce error associated with estimating a mPMI [30]. By researching different populations of flies and their ecological similarities and differences, forensic entomologists can partially explain the variation associated with the consumption phase of the post-colonization interval and infer a mPMI [5].

Temperature and tissue type can both contribute to error associated with mPMI estimates based on larval growth or stage duration, by impacting the development of the immature flies. By

assessing larval growth and development under various conditions one can help determine all potential factors leading to mPMI errors. Accounting for this error will support the use of this evidence in a court of law and meet the Daubert standard within the United States (Daubert et al., v. Merrell Dow Pharmaceuticals 1993 (509 U.S. 579)). By accounting for variation (i.e., error) associated with mPMI estimates, forensic entomologists can better understand the limitations of their methods. Validation of laboratory development data with field studies should accompany all new development data [31,36,37]. Doing so will result in establishing error rates with estimates of mPMI assigned to human death investigations.

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